IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	10/080,713	Confirmation No.: 9155
Applicant	:	Alan Colman et al.	
Filed	:	February 25, 2002	
TC/A.U.	:	1600/1632	
Examiner	:	Thaian N. Ton	
Title	:	Method of Preparing a Somatic Cell for Nuclear Transfer	
Docket No.	:	10758.105015	
Customer No.	:	20786	

June 23, 2005

DECLARATION OF DAVID L. AYARES, Ph.D.

1. My name is David L. Ayares. I am a cell biologist and embryologist working primarily in the field of nuclear transfer and genetic modification of donor cells to develop transgenic cloned animals for human therapeutic applications.

2. I attended Purdue University where I obtained a B.S. in Microbiology in 1982. I then obtained a Ph.D. in mammalian genetics from the University of Illinois Medical Center in 1987 where I analyzed the molecular biology of homologous recombination and DNA mismatch repair in mammalian cells. Following my graduate studies, I was a Postdoctoral Fellow in the Department of Biology at the Massachussetts Institute of Technology where I studied the molecular biology of protein degradation in mammalian cells.

3. Since 1990, I have been working in the biotechnology/ pharmaceutical industry. I worked at Abbott Laboratories as a Senior Research Cell/Molecular Biologist where I directed transgenic animal research and focused on the use of homologous recombination to generate genetic modifications in human cell lines and mouse embryonic stem cell lines until 1995. From 1995-1997, I worked at Baxter Healthcare as a research scientist in the gene therapy division focusing on gene delivery for gene therapy and the development of adenovirus-based vector systems, as well as novel helper cell lines, and transgenic animal models for *in vivo* gene therapy.

4. In 1997, I joined PPL Therapeutics, Inc., first as the Head of Molecular and Cell Biology and then as the Chief Operating Officer and Vice President of Research, where I directed all administrative and scientific aspects of the US Division of PPL Therapeutics. I managed the research of a multi-disciplinary team of scientists, focused on the development of novel vectors, cell lines, and transgenic animals (including mice, rabbits, pigs and cows) for applications in xenotransplantation, stem cells, and the production of therapeutic human proteins in milk.

5. I am currently the Chief Executive Officer and Chief Scientific Officer of Revivicor, Inc. (formerly PPL Therapeutics, Inc.), where I direct all administrative and scientific aspects of the company. Revivicor focuses on regenerative medicine including the production of genetically modified animals to provide human compatible cells, organs and tissues for use in human transplant surgery (xenotransplantation), as well as the production of therapeutic fully human polyclonal antibodies derived from genetically modified animals for a variety of infectious disease applications.

Somatic Donor Cells

6. I have read and I understand the article Oback B & Wells D. (2002) Donor cells for Nuclear Cloning: Many are Called, but Few are Chosen. Cloning Stem Cells 4(2): 147-168). I have in particular studied the data presented in Table 1 of the Oback paper.

7. Table 1 lists experimental cloning efficiencies of a range of somatic donor cells. Several of the somatic cell types have a reported "0" cloning efficiency. From that information, I understand that the Examiner has concluded that not all somatic donor cells can be used in all animal species to produce a live animal. I also understand that the Examiner suggests that "the state of the art clearly teaches that the donor cells to be used in somatic NT methods are neither predictable nor routine for different species."

8. Based on my experience in this field, I disagree with the Examiner's interpretation of the Oback paper. I know of no somatic cell that cannot be cloned as long as it has a normal karyotype.

9. The concept of cloning efficiency is a reflection of the ratio of number of attempts versus the number of successes (i.e, viable clones obtained). It illustrates a very well known and simple observation that certain somatic cells produce more viable embryos after transfer than others. Since some somatic cells produce viable embryos with a lower efficiency than others, when it is desired to use such lower efficiency cells, more embryos should be transferred.

10. The general technique of nuclear transfer is well known. When using a new donor cell type for somatic cell nuclear transfer, the worker simply has to recognize that it is a numbers game, and one must repeat the experiment using the well known techniques until success is achieved.

11. There is no fundamental reason that prevents any somatic cell with a normal karyotype from acting as a nuclear donor. If a viable embryo is not obtained, then the researcher simply needs to conduct more embryo transfers until viability is achieved. Repeating the experiment enough times to achieve success does not require a special or extra technique, it simply requires that you carry out the experiment more times. It can routinely require hundreds of embryo transfers to produce a viable cloned animal.

12. In my experience, while in most cells it is sufficient to transfer between 200-500 embryos to obtain a viable offspring, on two occasions we have carried out as many as 2000 embryo reconstructions to obtain a viable cloned offspring. The transfer of as many as 2000 embryos can take as little as two weeks to perform on a routine basis.

13. Indeed, Table 1 of Oback reports the cloning efficiency of fibroblast cells. Fibroblasts have been the most commonly used donor cell for nuclear transfer. Oback reports a cloning efficiency for fibroblasts of between 0.05% and 1.2%. A cloning efficiency of 0.05% represents 1 viable clone (i.e. live birth) per 2000 embryos transferred.

14. The cloning efficiency data generated by Oback based on the information presented in Table 1 are inaccurate due to the phenomenon of sampling error. When a sample size is insufficiently small, it does not represent the population from which it is taken and can thus lead to false negative results.

15. Table 1 omits the most important numbers, which are the number of embryos transferred to surrogate mothers in each experiment. The number of embryos transferred is a critical number because it relates directly to cloning efficiency. As discussed above, cloning efficiencies of 0.05% have been reported, which represents 2000 embryos transferred for 1 live birth. Table 1 provide "blastocyst" numbers as percentages calculated on the basis of numbers of reconstituted NT embryos. The Table also presents "implantation" numbers as percentages calculated on the basis of numbers of embryos transferred.

16. I have referred to the citations in the Table and calculated the actual number of embryos transferred. In particular, references 8 (Wakayama and Yanagimachi (2001). Molecular Reproduction and Development, 58, 376-383) and 15 (Wakayama *et al.* (1998). Nature, 394, 369-374) of Table 1 provide data for murine mature sertoli cells, murine lymphoctytes, murine macrophages, murine leukocytes and murine neurons as donor cells, for which the cloning efficiency is reported in Oback as "zero". My calculation of the number of embryos transferred for each of those cell types is listed below.

Tissue Origin	***Cell Type***	***Species***	***Embryos Transferred***	***Reference***
Adult				
Testis	Mature Sertoli	Mouse	59 (total #)	8
Thymus	Lymphocyte	Mouse	0	8
Peritoneal Cavity	Macrophages	Mouse	52/25 (total #: female/ male)	8
Spleen	Leukocytes	Mouse	11/8 (total #: female/ male)	8
Brain	Neuron? Glia?	Mouse	46 (total #)	15

17. As evident in the above table, the cloning efficiencies for the cell types listed were calculated based on 0-75 total embryo transfers per cell type. Thus, the sample number was too small to draw any conclusions on the "clonability" of these cell types.

18. Indeed, for some cell types used in studies cited in Table 1 cloning efficiency was originally reported as zero, however, more recent publications have clearly demonstrated that viable clones are produced.

19. For example, in Table 1, murine neuronal cells were reported to have a "0" cloning efficiency. However, Yamazaki et al reported that when neural cell nuclei were transferred into enucleated oocytes, 5.5% of the reconstructed oocytes developed into normal mice (Yamazaki, et al, Assessment of the developmental totipotency of neural cells in the cerebral cortex of mouse embryo by nuclear transfer. *Proceedings of the National Academy of Sciences*, November 20, 2001, Vol 98, pages 14022-14026).

20. Also in Table 1, murine lymphocytes from the thymus and leukocytes from the spleen were reported to have a "0" cloning efficiency. However, Hochedlinger & Jaenisch (*Nature* 2002 Feb 28 :Monoclonal mice generated by nuclear transfer from mature B and T donor cells" 415(6875):1035-8. Epub 2002 Feb 10) reported the generation of monoclonal mice by nuclear transfer from mature T cells (lymphocytes from the thymus) and B cells (leukocytes from the spleen). In particular, the authors state "This is an unequivocal demonstration that a terminally differentiated cell can be reprogrammed to produce an adult cloned animal."

21. As an example of cloning new, as yet never tried, cell types, we recently set out to clone pigs using a donor cell type, endothelial cells, which had never been used for cloning. Our first round of new experiments resulted in the successful production of cloned pigs from endothelial cells. We transferred 341 embryos into one surrogate mother. This transfer resulted in pregnancy and delivery of three healthy piglets on day 119 of gestation. Thus, our cloning efficiency for endothelial cells was 0.9%, which is within the range reported for other cell types. This represents a typical strategy that one skilled in the art would employ to clone a new cell type.

Homologous Recombination

22. Homologous recombination is a natural event that occurs in all cells. Homologous recombination underlies many biological pathways. The recombination machinery has been well conserved throughout evolution as an essential component of cell survival. In cells, homologous recombination is a DNA maintenance pathway that protects chromosomes against damage affecting both DNA strands, such as double strand breaks or interstrand crosslinks. In addition to its maintenance role, homologous recombination underlies numerous other biological events. For example, it is involved in meiotic crossovers, which are responsible for the rearrangement of alleles, as well as necessary for proper chromosome segregation. It is also responsible for mating type switching, epitope class switching, mutations, translocations, gene rearrangements and evolution in cells.

23. Homologous recombination is the leading technology in rational genome engineering. Many fundamental discoveries in biology have taken place with the help of homologous recombination. Scientists have exploited the cell's endogenous machinery to use homologous recombination to manufacture site directed insertions, deletions or replacements of DNA in a living organism for the past 20 years.

24. Therefore, all cells can successfully undergo homologous recombination because it is an essential mechanism required for all viable cells.

25. During the interview with the Examiners, the issue was raised regarding whether cells other than fibroblasts could be targeted since other cell types may not have equivalent proliferative potential. As discussed with the Examiners, it was clearly feasible as of the filing date of the present application to obtain and screen for homologous recombination in cell types that do not have high proliferative potential. This fact was first demonstrated in 1989 by Zimmer and Gruss (Nature 338: 150-153) and 1990 by Jasin et al (Genes & Development (1990) 4: 132-166).

26. Zimmer & Gruss described a rapid polymerase chain reaction (PCR)-based screening method to identify homologous recombination events in mouse embryonic stem cells without prolonged culture and cell expansion. This paper was hailed as a significant advance since, at that time, relatively few mouse embryonic stem cell lines could be cultured long term without undergoing differentiation. This was problematic since one could identify homologous recombination events after prolonged culture but since the embryonic stem cells had differentiated they were not useful for production of germ line chimeras. Zimmer's PCR assay (in which a diagnostic PCR product is amplified using one primer within a marker gene within the targeting vector, and a second primer located in the genomic target sequence outside the region of homology), did not require growth of embryonic stem cell clones from single colonies to one million cells (as required prior to the Zimmer publication) but rather could be used to identify homologously recombined clones as soon as they had formed.

27. The use of Fluorescent Activated Cell Sorting (FACS) to rapidly detect homologous recombination events in transfected cells has facilitated genetic targeting in all cell types. The Jasin paper (Genes & Development (1990) 4: 132-166) was a fundamental publication that established the importance of this strategy of selection for gene targeting. The Jasin paper reported a 700-fold enrichment for homologous versus nonhomologous integration events by using FACS to select the targeted cells.

28. Therefore, as early as 1989, several methods, including PCR-based and FACS-based techniques, were known to those skilled in the art, which could be used to detect targeted integration events after homologous recombination within 3-5 days. These methods allowed one to identify targeted clones, in a variety of cell types, without prolonged in vitro growth and expansion. It is clearly not a pre-requisite that cells to be used for homologous recombination and subsequent nuclear transfer must have high or even moderate proliferative potential.

29. I declare further that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, imprisonment, or both, under Section 1001, Title 18, United States Code, and that willful false statements may jeopardize the validity of this application and any patent issuing thereon.

6/22/05
Date

David L. Ayares, Ph.D.